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Mutation Research 596 (2006) 128–136
Fundamental and Molecular Mechanisms of Mutagenesis
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Exogenous 8-oxo-dG is not utilized for nucleotide synthesis but enhances the accumulation of 8-oxo-Gua in DNA through error-prone DNA synthesis

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Received 20 October 2005; received in revised form 14 December 2005; accepted 22 December 2005
Available online 10 February 2006

Abstract

7,8-Dihydro-8-oxoguanine (8-oxo-Gua) and its nucleoside in cytosol are derived from the repair of oxidative DNA and the cleanup of oxidatively damaged DNA precursors, respectively. While the harmful effects of 8-oxo-Gua present in DNA have been studied extensively, few have reported its effects on cytosolic function. Our previous study showed that the addition of 8-oxo-dG to culture media caused an accumulation of 8-oxo-Gua in nuclear DNA in several leukemic cells including KG-1, which lack 8-oxoguanine glycosylase 1 (OGG1) activity due to mutational loss. However, the mechanism underlying 8-oxo-Gua level increases in DNA has not been addressed. In this study, we elucidated the metabolic fate of 8-oxo-Gua-containing nucleotide and the effect of exogenous 8-oxo-dG on DNA synthesis in KG-1 cells. We found that 8-oxo-dGMP was rapidly dephosphorylated to 8-oxo-dG rather than phosphorylated to 8-oxo-dGDP, thus indicating that 8-oxo-Gua-cotaining molecule is not used as a substrate for DNA synthesis in KG-1 cells. In fact, radiolabled 8-oxo-dG was incubated but radioactivity was not detected in nuclear DNA of KG-1 cells, showing that 8-oxo-dG is not directly incorporated into DNA. Interestingly, the activity of DNA polymerase β, which synthesize DNA with low fidelity increased in KG-1 cells treated with 8-oxo-dG, whereas the expression of DNA polymerase α decreased. In addition, the accumulation of 8-oxo-Gua in KG-1 DNA was completely inhibited by a specific inhibitor of DNA polymerase β. Thus, our findings address that the insertion of 8-oxo-dG into KG-1 DNA is not due to the direct incorporation of exogenous 8-oxo-dG, but rather to the inaccurate incorporation of endogenous 8-oxo-dGTP by DNA polymerase β. It further suggests that 8-oxo-dG in the cytosol may function as an active molecule itself and perturb the well-defined DNA synthesis.

Keywords: DNA polymerase β; OGG1; Oxidative stress; 8-oxo-deoxyguanosine

1. Introduction

7,8-Dihydro-8-oxoguanine (8-Oxoguanine; 8-oxo-Gua) is one of most abundant oxidative DNA adducts and thus is used as a marker of oxidative DNA damage [1]. 8-oxo-Gua in DNA is promutagenic since it

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doi:10.1016/j.mrfmmm.2005.12.004

promotes the incorporation of dATP instead of dCTP opposite this lesion during replication, inducing a GC to TA transversion [2,3]. 8-oxo-Gua in DNA also has an oxidative damage-inducing effect on the neighborhood bases due to its electron-rich nature [4,5]. In addition, 8-oxo-Gua in DNA can induce the formation of reversible topoisomerase1-linked DNA single-stranded breaks [6,7]. Therefore, 8-oxo-Gua in DNA is a harmful residue that can lead to carcinogenesis and potentially perturb cell functions to cause even cell death. However, cells are equipped with repair systems to removes 8-oxo-Gua in DNA by OGG1 (which repairs 8-oxo-Gua:C) [8], OGG2 (8-oxo-Gua:A) [9], and NTH1 (8-oxo-Gua:G) [10]. In addition, nucleotide excision repair [11] and endonuclease [12] are found to finally generate a mononucleoside form, 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG).

In contrast to 8-oxo-Gua in DNA, we do not have information on the actions of cytosolic 8-oxo-Gua-containing nucleoside, for example, 8-oxo-dG. In fact, nucleosides of purine and pyrimidine analogues have been introduced as chemotherapeutic agents, which are 2-chloro-2′-deoxyadenosine (CdA), 2-fluoro-9-β-D-arabionofuranosyladenine (F-AraA), 1-β-D-arabiofuranosylcytosine (AraC), 2′,2′-difluorodeoxycytidine (dFdC), and 9-β-D-arabionofuranosylguanine (AraG) [13,14]. These analogues are taken up into cells and phosphorylated to nucleoside triphosphates. The phosphorylated analogues can exert cytotoxic activity by being incorporated into DNA and RNA and altering their structures, by interfering with various enzymes involved in the synthesis of nucleic acids such as DNA polymerases and ribonucleotide reductase, or by modifying the metabolisms of physiological nucleosides [15]. These actions result in the inhibition of synthesis of nucleic acids and eventually cell death. Therefore, we hypothesized that 8-oxo-dG can be also cytotoxic possibly due to its incorporation into DNA.

Our previous study showed that exogenous 8-oxo-dG added to KG-1 cell (a human acute leukemia cell) in culture, caused G1 cell cycle arrest, apoptotic cell death, and accumulation of 8-oxo-Gua in nuclear DNA [16,17]. This cell is deficient in OGG1 (8-oxoguanine glycosylase 1, a repair enzyme of 8-oxo-Gua) activity and thus is incapable of removing 8-oxo-Gua from DNA. Other leukemic cell lines with low OGG1 activity, i.e. H9, CEM-CM3, and Molt-4, were also found to have similar cytotoxic responses to exogenous 8-oxo-dG, whereas U937 or Jurkat cells with normal OGG1 activity were not [17]. The cytotoxicity induced by 8-oxo-dG could be explained due to a low capacity of OGG1 to cope with an accumulation of 8-oxo-Gua in DNA.

However, little information is available regarding the mechanism by which exogenous 8-oxo-dG increases the level of 8-oxo-Gua in DNA. In this study, we examined whether 8-oxo-Gua-containing molecules such as 8-oxo-dGMP is utilized as a substrate for the enzymes involving in DNA synthesis and then directly incorporated into DNA, or whether 8-oxo-dG indirectly affects DNA synthesis. We found that 8-oxo-dGMP was not phosphorylated into 8-oxo-dGDP but instead, dephosphorylated to 8-oxo-dG and confirmed that exogenous 8-oxo-dG was not directly incorporated into DNA. However, the KG-1 treated with exogenous 8-oxo-dG showed the increase of DNA polymerase β activity and the increase of 8-oxo-Gua in DNA by added 8-oxo-dG was blocked by dideoxythymidine, a DNA polymerase β inhibitor. These results suggest that 8-oxo-dG is not metabolized and itself can cause the accumulation of 8-oxo-Gua in DNA through the activation of non-replicative DNA synthesis, which may enhance the inaccurate incorporation of endogenous 8-oxo-dGTP into DNA.

2. Materials and methods

2.1. Materials and cell lines

2′-Deoxyguanosine (dG), 7,8-dihydroxy-8-oxo-2′-deoxyguanosine (8-oxo-dG), deoxyguanosine 5′-triphosphate (dGTP), 2′,3′-dideoxythymidine, guanylate kinase, nucleotidase, purine nucleoside phosphorylase and nuclease P1 were obtained from Sigma. 5′-[α-^{32}P]-dGTP (800 Ci/mmol), [1′,2′-^{3}H]-dGTP (40 Ci/mmol), and deoxy[methyl-^{3}H] thymidine 5′-triphosphate (dTTP) were purchased from Amersham Pharmacia Biotech. Alkaline phosphatase was purchased from Roche Molecular Biochemicals. U937 and KG-1 cells were obtained from the American Type Culture Collection and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in 5% CO_2.

2.2. Preparation of radiolabeled 8-oxo-dG and 8-oxo-dGMP

^{3}H-labeled 8-oxo-dG was prepared by the oxidation of ^{3}H-labeled-dG according to the modified procedure [18]. α-^{32}P-labeled 8-oxo-dGMP was prepared by the oxidation of α-^{32}P-labeled dGTP and its subsequent dephosphorylation of oxidized dGTP according to the previously described procedure [19,20]. All radiolabeled nucleosides and nucleotides were purified by high performance liquid chromatography (HPLC).

2.3. Guanylate kinase assay

8-oxo-dGMP was tested whether this oxidized nucleotide can be a substrate for guanylnate kinase using crude cell extract of KG-1 cells as an enzyme source. To prepare crude cell extract, KG-1 cells (2×10^{8}) was suspended in a lysis buffer

(300 μl) containing 20 mM Tris–HCl, pH 7.4, 0.5 mM PMSF (phenylmethanesulfonyl fluoride) and 0.5 μg/ml of leupeptin and pepstatin A, and lysed by three cycles of freezing and thawing. After treatment with Norit A to remove endogenous nucleotides [21], the lysate was cleared by ultracentrifugation at 100,000 × *g* for 1 h at 4 °C and the supernatant was used as a crude cell extract. [α-^{32}P]-dGMP or [α-^{32}P]-8-oxo-dGMP (0.4 mM each) was incubated at 37 °C for up to 4 h with 4 μg crude cell extract or 1 mU guanylate kinase [19] in a reaction mixture (5 μl) containing 0.25 M KCl, 20 mM $MgCl_2$, 5 mM ATP, 0.1 M Tris–HCl, pH 8.0. At the times indicated, aliquots (0.5 μl) were withdrawn from the reaction and mixed with 1 μl of 50 mM EDTA to terminate the reaction. Thin layer chromatography was used to analyze the reaction products by spotting the aliquot/EDTA mixtures on PEI-cellulose F plate (Merck) and developing the plate in 1 M LiCl. The amounts of reaction products were quantified by measuring the densities of the nucleotide spots on the autoradiographs using a microcomputer imaging device (Imaging Research Inc.).

2.4. Nucleotidase assay

Crude cell extract was prepared as described above. [α-^{32}P]-dGMP or [α-^{32}P]-8-oxo-dGMP (50 μM each) was incubated at 37 °C with 0.5 μg crude cell extract, 2 mU alkaline phosphatase or 2 mU nucleotidase in a reaction mixture (5 μl) containing 4 mM $MgCl_2$, 80 μg/ml BSA, 8 mM dithiothreitol, 2% glycerol and 20 mM MOPS-NaOH, pH 6.5 [19]. After incubation for various times ranging from 15 to 60 min, reaction products were analyzed by thin layer chromatography as described above.

2.5. Measurement of 8-oxo-Gua in nuclear DNA

8-oxo-Gua level in nuclear DNA was measured according to the procedure described before [5]. Briefly, cells (5×10^6) was cultured without or with 2 μM dideoxythymidine for 3 h and then further without or with 400 μM 8-oxo-dG for at 37 °C. Nuclear DNA was isolated by NaI extraction technique using a cDNA extractor WB kit according to the manufacturer's instructions (Wako Pure Chemical Industries Ltd.). Nuclear DNA was hydrolyzed to the nucleoside level and the separation of nucleosides was achieved using a reverse phase Supelcosil LC-18-S column (Supelco, 5 μm, 4.6 mm × 250 mm) at a flow rate of 0.5 ml/min. The mobile phase was 50 mM NaH_2PO_4/10% methanol. Unmodified nucleosides were monitored by a Model 112 UV detector (Gilson) set at 254 nm. 8-oxo-dG was determined with a Coulochem II electrochemical detector (ESA).

2.6. DNA incorporation of labeled 8-oxo-dG

Cells (5×10^6) were incubated with 400 μM dG plus 2 μCi of [1′,2′-^{3}H]-dG or 400 μM 8-oxo-dG plus 2 μCi of [1′,2′-^{3}H]-8-oxo-dG for 48 h and then washed with cold PBS. Intracellular uptake was detected by measuring the radioactivity of harvested cells. To detect the incorporation into DNA, nuclear DNA was isolated by NaI extraction technique using a cDNA extractor WB kit as described above and its radioactivity was measured by liquid scintillation counting [22,23].

2.7. RT-PCR (reverse transcription coupled polymerase chain reaction) of DNA polymerase α

Expression of DNA polymerase α was observed in cells treated with 8-oxo-dG as follows. Cells (5×10^6) were cultured without or with 8-oxo-dG (200 or 400 μM) at 37 °C. At 24 or 48 h after treatment, total RNA was extracted from cells using the guanidine precipitation method with TRIzol according to the manufacturer's instructions (Molecular Research Center). cDNA was synthesized from the total RNA using 1st strand cDNA synthesis kit according to the manufacturer's instructions (Roche Applied Science). Using the cDNA, PCR was done toward the two genes, DNA polymerase α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR was programmed to operate sequentially with an initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s/annealing at 60 °C for 30 s/extension 72 °C for 1 min, and a final extension at 72 °C for 7 min. The primers used were; DNA polymerase α – forward, GAGCTTATGCTGGAGGCTTG and reverse, CACCTTTCTGCCTCCTTGAG; and GAPDH – forward, 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and reverse, 5′-CATGTGGGCCATGAGGTCCACCAC-3′. PCR products were separated by electrophoresis in a 1.2% agarose gel and visualized by ethidium bromide staining.

2.8. Western blotting of DNA polymerase β

The protein expression level of DNA polymerase β was examined using the nuclear extract of 8-oxo-dG-treated cells. Cells (5×10^6) were cultured without or with 8-oxo-dG (200 or 400 μM) at 37 °C. At 24 or 48 h after treatment, cell pellet was suspended for 10 min at 4 °C in 10 volumes of a hypotonic buffer A containing 20 mM Tris–HCl, pH 7.8, 0.2 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM DTT, 0.8 mM Na_3VO_4 and 0.4 mM PMSF. Cells were then lysed by mixing with 1/10 volume of 6% NP-40 and vigorously vortexed for 10 s. After centrifugation at 10,000 × *g* for 5 min, the pellet obtained was mixed for 30 min at 4 °C with a high salt buffer which is the buffer A but containing 5% glycerol, 400 mM NaCl, and 1 μg/ml leupeptin and pepstatin A. After centrifugation at 20,000 × *g* for 10 min, the supernatant obtained was used as a nuclear extract. After measurement of protein concentration in supernatant, the same protein amount was subjected to SDS-PAGE. Western blotting was performed following standard procedures using a primary antibody of mouse anti-human DNA polymerase β (Neomarkers) or rabbit anti-human CREB (Santa Cruz).

2.9. DNA polymerase β assay

DNA polymerase β was assayed in the nuclear extract of 8-oxo-dG-treated cells. Cells were treated and the nuclear

extracts were prepared as described above. The enzyme activity was assayed by measuring the amount of [methyl-^{3}H]-thymidine 5′-triphosphate (dTTP) incorporated into an acid-insoluble material [24,25]. An assay mixture (25 μl) contained 5 μg of nuclear extract, 20 μg/ml poly(dA)-oligo(dT)$_{12–18}$, 100 mM NaCl, 4 mM *N*-ethylmaleimide (a SH blocker), 0.5 mM $MnCl_2$, 80 μM dTTP (including 2 μCi [methyl-^{3}H] dTTP) and 100 mM Tris–HCl, pH 8.8. After incubation for 30 min at 37 °C, reaction was terminated by adding 0.5 ml of cold 10% trichloroacetic acid (TCA) containing 0.2% sodium pyrophosphate and then kept for 10 min at 4 °C. The reaction mixture was put onto a pre-soaked GF/C filter (Whatman), which was washed with 2% TCA containing 0.02% sodium pyrophosphate and then with 100% ethanol. The filter was then dried and radioactivity of acid insoluble material on the filter was measured.

3. Results

3.1. 8-oxo-dGMP was dephosphorylated into 8-oxo-dG but not phosphorylated into 8-oxo-dGDP

It has been reported that treatment of exogenous 8-oxo-dG caused the accumulation of 8-oxo-Gua in nuclear DNA in KG-1 which is deficient in OGG1 activity, but not in U937 which has the normal enzyme activity [16]. However, it has been already reported that guanylate kinase which phosphorylates dGMP into dGDP, does not use 8-oxo-dGMP as a substrate, and thus this enzyme acts as a gate-keeper to prevent the incorporation of 8-oxo-dG into DNA [19,26]. In order to explain the 8-oxo-dG-induced accumulation of 8-oxo-Gua in KG-1 DNA, we assumed that the property of guanylate kinase in KG-1 cells is altered to make 8-oxo-dGDP from 8-oxo-dGMP and thus permits the direct incorporation of 8-oxo-dG into the DNA of these cells. Therefore, we tested whether [^{32}P]-radiolabeled dGMP or 8-oxo-dGMP was changed into nucleoside diphosphate by cell extracts of KG-1 and U937 cells. As expected, unmodified dGMP was phosphorylated to dGDP and further to dGTP by both of the cell extracts (Fig. 1A), and this phosphorylation to dGDP was confirmed by purified guanylate kinase (Fig. 1A). However, 8-oxo-dGMP was not phosphorylated to 8-oxo-dGDP by guanylate kinase or cell extracts, but rather was dephosphorylated to 8-oxo-dG even in the presence of ATP by the cell extracts (Fig. 1A). A fast-moving radioactive spot was identified to be an inorganic orthophosphate by incubating 8-oxo-dGMP with alkaline phosphatase (data not shown). This indicates that 8-oxo-dGMP is predominantly degraded to 8-oxo-dG in KG-1 as well as in U937 cells.

Next, we compared the substrate specificities of dGMP and 8-oxo-dGMP for dephosphorylation. [^{32}P]-radiolabeled dGMP or 8-oxo-dGMP was incubated with purified nucleotidase or cell extract of U937 or KG-1 cells in the absence of ATP. dGMP was found to be dephosphorylated by purified nucleotidase and cell extracts. 8-oxo-dGMP was also dephosphorylated into 8-oxo-dG by both extracts but much less by purified nucleotidase (Fig. 1B). Interestingly, however, 8-oxo-dGMP was even more dephosphorylated into 8-oxo-dG by both cell extracts than dGMP was into dG (Fig. 1B). These results suggest that 8-oxo-dGMP in vivo can be extensively degraded to 8-oxo-dG presumably by some specific phosphatase(s). From these results, we know that 8-oxo-Gua-containing nucleotide in both KG-1 and U937 cells is converted to a dephosphorylated nucleoside form and thus has no chance to be used as a substrate for DNA synthesis. Therefore, it highly suggests that the increase of 8-oxo-Gua level in KG-1 DNA by exogenous 8-oxo-dG is not induced by the direct incorporation of added 8-oxo-dG.

3.2. 8-oxo-dG was not incorporated into DNA

In order to confirm that exogenous 8-oxo-dG is not used for DNA synthesis, 8-oxo-dG together with [^{3}H]-radiolabeled 8-oxo-dG was incubated with KG-1 and U937 cells and then both radioactivity and 8-oxo-Gua level were determined in the DNAs isolated from these cells. As shown in Table 1, the level of 8-oxo-Gua in nuclear DNA was significantly much higher in KG-1 than in U937 cells as previously reported [16], but the radioactivity of DNA was very little in both U937 and

Table 1
Incorporation of 8-oxo-dG into DNA

	U937	KG-1
Intracellular uptake		
dG	0.71 (±0.06)	0.69 (±0.05)
8-oxo-dG	0.35 (±0.03)	0.39 (±0.02)
Nuclear DNA		
dG	0.13 (±0.03)	0.09 (±0.02)
8-oxo-dG	5.2×10^{-5} ($\pm 1 \times 10^{-5}$)	3.4×10^{-5} ($\pm 8 \times 10^{-6}$)
Increased 8-oxo-dG/10^5 dG	0.22 (±0.03)	1.22* (±0.44)

Cells (5×10^6) were incubated with 400 μM of dG or 8-oxo-dG including 2 μCi of [1′,2′-^{3}H]-dG or [1′,2′-^{3}H]-8-oxo-dG, respectively, for 48 h and then washed with cold PBS. Intracellular uptake of dG or 8-oxo-dG was measured by counting the radioactivities (μCi) of harvested cells. Incorporation into DNA was measured by the radioactivities of DNAs isolated from cells treated with dG or 8-oxo-dG. The increased amounts of 8-oxo-dG in DNA (50 μg) were calculated by substracting the value before from after treatment of 8-oxo-dG. Details are described in Section 2.

* $P < 0.05$; significant difference.

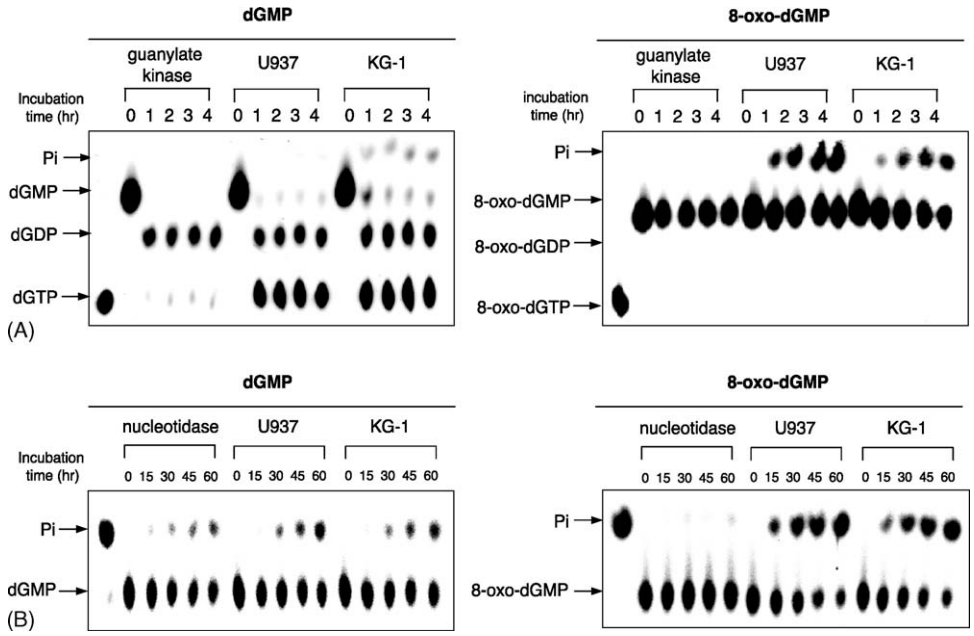

Fig. 1. 8-oxo-dGMP is rapidly degraded to 8-oxo-dG rather than phosphorylated to 8-oxo-dGDP. (A) 0.4 mM [α-^{32}P]-dGMP or [α-^{32}P]-8-oxo-dGMP were incubated with either 1 mU of purified guanylate kinase or 4 μg of crude cell extracts from U937 or KG-1 cells at 37 °C in the presence of ATP. (B) 50 μM of [α-^{32}P]-dGMP or [α-^{32}P]-8-oxo-dGMP were incubated with either 2 mU of purified nucleotidase or 0.5 μg of crude cell extracts from U937 or KG-1 cells at 37 °C in the absence of ATP. Reaction mixtures were analyzed by thin layer chromatography. Details are described in Section 2.

KG-1 cells while the radioactivity of intracellular uptake was high in both cells. The radioactivity detected in intracellular compartment is lower with 8-oxo-dG than with dG, suggesting that 8-oxo-dG is readily excreted. Overall, the results clearly indicate that 8-oxo-dG is taken up into cytoplasm, but is not incorporated into DNA and also that the accumulation of 8-oxo-dGua in KG-1 DNA is not attributed to the direct incorporation of 8-oxo-dG into DNA.

3.3. Non-replicative DNA synthesis was enhanced by treating KG-1 cells with 8-oxo-dG whereas replicative DNA synthesis was inhibited

The above findings on the metabolic fates of 8-oxo-dG and 8-oxo-dGMP addressed that increase in 8-oxo-Gua level in DNA is not resulted from incorporation of exogenous 8-oxo-dG. There are at least two alternative explanations for the accumulation of 8-oxo-Gua in DNA. One is that exogenous 8-oxo-dG may trigger oxidative stress and thus produce oxidatively modified DNA adducts. However, this explanation is excluded by a previous finding [17], showing that *N*-acetylcysteine was unable to block apoptotic cell death induced by 8-oxo-dG. The other possibility is that cellular responses to 8-oxo-dG treatment alter normal DNA synthesis and stimulate the incorporation of endogenous oxidized nucleotides, including 8-oxo-dGTP. In order to test the latter possibility, we examined the expression levels of DNA polymerases. The 8-oxo-dG treatment decreased mRNA level of DNA polymerase α in KG-1 but not in U937 cells (Fig. 2A). DNA polymerase α is one of replicative DNA polymerases with high fidelity during S phase and its gene is one of E2F1-regulated genes during entry into S phase [27,28]. Therefore, this result reflects G1 arrest in KG-1 cells induced by 8-oxo-dG [29].

In contrast, the expression of DNA polymerase β protein determined by Western blotting was not changed in KG-1 or U937 cells by treatment with 8-oxo-dG (Fig. 3B). DNA polymerase β is a non-replicative DNA polymerase and is expressed constantly throughout the cell cycle [30,31]. However, the enzymatic activity of DNA polymerase β increased in KG-1 cells treated with 8-oxo-dG (Fig. 2B), indicating that exogenous 8-oxo-dG enhances the activity of non-replicative DNA synthesis in KG-1 cells. The basal levels of expression and activity of DNA polymerase β as well as CREB (cAMP response element binding protein), which is one of transcriptional factor to control the mRNA level of DNA polymerase β, were also higher in KG-1 than in U937 cells (Fig. 2A and B).

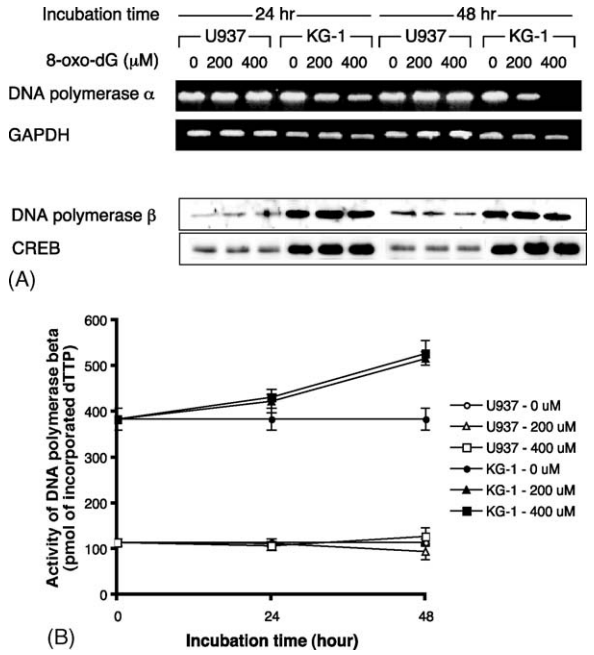

Fig. 2. The activity of DNA polymerase β, but not its expression level, increases after treating KG-1 cells with 8-oxo-dG. (A) Total mRNA and nuclear protein were isolated from 8-oxo-dG-treated U937 and KG-1 cells. mRNA level of DNA polymerase α and protein level of DNA polymerase β were analyzed by RT-PCR and Western blot, respectively. GAPDH mRNA and CREB (cAMP response element binding protein) protein were used as a control. (B) 5 μg of nuclear extracts from U937 or KG-1 cells treated with 8-oxo-dG for the indicated times were then incubated with [methyl-^{3}H] dTTP and poly(dA)-oligo(dT)$_{12–18}$ for 30 min at 37 °C. Reaction mixtures were spotted on GF/C filter and acid insoluble radioactivity was measured. The activity of DNA polymerase β was defined as the amount of dTTP incorporated into synthetic template-primers. Details are described in Section 2.

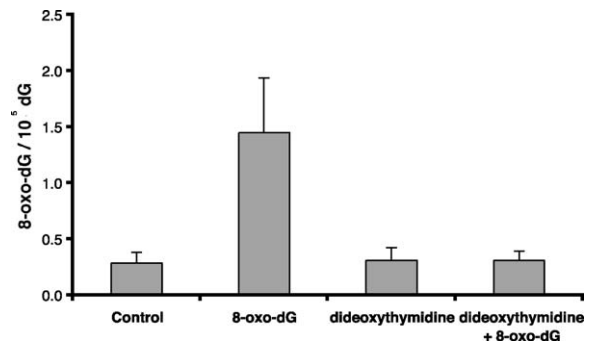

Fig. 3. The accumulation of 8-oxo-dGua in DNA is abolished by an inhibitor of DNA polymerase β in KG-1 cells. KG-1 cells were pre-incubated with dideoxythymidine, a specific inhibitor of DNA polymerase β for 3 h and treated with 8-oxo-dG. At 48 h after this treatment, nuclear DNA was extracted and the amount of 8-oxo-dG in DNA was quantified using HPLC-ECD. Details are described in Section 2.

The above finding raises a possibility that the accumulation of 8-oxo-Gua in DNA is mediated by DNA polymerase β because this enzyme is known to easily incorporate abnormal dNTP including 8-oxo-dGTP [32,33]. To test this hypothesis, KG-1 cells were pretreated with dideoxythymidine, a specific DNA polymerase β inhibitor, prior to incubation with 8-oxo-dG. Pre-exposure of KG-1 cells to dideoxythymidine blocked the increase in 8-oxo-Gua level in KG-1 DNA (Fig. 3), strongly supporting this possibility that the accumulation of 8-oxo-Gua in KG-1 DNA is due to the enhanced incorporation of endogenous 8-oxo-dGTP mediated by the activation of DNA polymerase β. Overall, these findings suggest that exogenous 8-oxo-dG in KG-1 cells causes an imbalance between replicative and non-replicative DNA synthesis, and thus allows the cells to synthesize DNA in a low fidelity manner.

4. Discussion

In the present study, we observed that 8-oxo-dGMP was not phosphorylated to 8-oxo-dGDP but rather rapidly degraded to 8-oxo-dG in both KG-1 and U937 cells (Fig. 1), indicating that 8-oxo-dG has no chance to be salvaged for DNA synthesis. In addition, we failed to detect the radioactivity in the nuclear DNAs of both U937 and KG-1 cells after incubation with radiolabeled 8-oxo-dG (Table 1). Therefore, we can exclude a possibility that the previously observed accumulation of 8-oxo-Gua in DNA of OGG1-deficient KG-1 cells might be due to the direct incorporation of added 8-oxo-dG into DNA. However, we found a difference between KG-1 and U937 cells in response to exogenous 8-oxo-dG, which was the enhanced activity of DNA polymerase β only in KG-1 cells (Fig. 2). This enzyme has an error-prone DNA synthetic activity in non-replicative status by incorporating abnormal dNTP including 8-oxo-dGTP. Strikingly, pretreatment of KG-1 cells with dideoxythymidine, an inhibitor of DNA polymerase β abolished the accumulation of 8-oxo-Gua in DNA (Fig. 3). In contrast to DNA polymerase β, DNA polymerase α was down-regulated in KG-1 cells by 8-oxo-dG. This enzyme synthesizes DNA with high fidelity in DNA replication. Overall, these findings suggest that exogenous 8-oxo-dG in KG-1 cells disturbs normal DNA synthesis but enhances the error-prone DNA synthesis which incorporates endogenous 8-oxo-dGTP into KG-1 DNA. KG-1 cells with no OGG1 repair activity cannot remove 8-oxo-Gua from DNA, resulting in accumulation of 8-oxo-Gua in DNA.

8-oxo-dG in urine is used as a biological marker of oxidative stress [18]. Free 8-oxo-dG is derived either from the repair of oxidized DNA or from the contin-

ual dephosphorylation of 8-oxo-dGTP. MTH (8-oxo-dGTPase) hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP [20], which is then degraded to 8-oxo-dG presumably by a specific phosphatase with high affinity for 8-oxo-dGMP. Unmodified deoxynucleosides derived from DNA or from nucleotides are reutilized for nucleotide synthesis. However, 8-oxo-dG is not used for nucleotide synthesis because deoxynucleoside kinase (unpublished data) and guanylate kinase [19] cannot act on 8-oxo-Gua-containing molecules. Therefore, 8-oxo-dG with a hydrophilic nature, once it is produced, is readily excreted into urine without further metabolism and thus it has validity for a maker of oxidative stress. Overall, cells are equipped with several protective systems to prevent the reuse of mutagenic 8-oxo-Gua-containing molecules for DNA synthesis, reflecting the significance of 8-oxo-Gua in genotoxicity.

We previously observed that the 8-oxo-dG-treated KG-1 cells showed high level of 8-oxo-Gua in DNA and were destined to apoptosis, while under the same condition, 8-oxo-dG showed no effect on U937 cells with normal OGG1 activity in terms of the 8-oxo-Gua level in DNA and cell survival [16,17]. However, it still remains to be determined how non-metabolized 8-oxo-dG triggers cytotoxicity in KG-1 cells. Based on our findings, we propose that the cytotoxicity is, at least in part, due to the error-prone DNA synthesis mediated by DNA polymerase β. DNA polymerase β, a non-replicative polymerase, plays an essential role in gap-filling synthesis during base excision repair and also provides deoxyribonucleotide phosphate (dRP) lyase that excises dRP from the end of DNA incised by hydrolytic AP endonucleases [34]. Although some reports suggest that DNA polymerase β protects cells from carcinogenesis based on the finding that a variety of cancer cells show functional impairment of this enzyme due to mutation [35], many recent studies have reported detrimental functions of DNA polymerase β. This enzyme has no proofreading activity and poor ability to discriminate between normal and abnormal nucleotides. Thus, this enzyme carries out unfaithful DNA synthesis and thus induces spontaneous mutagenesis [36,37]. Accordingly, the high level of DNA polymerase β expression is suggested to lead to cancer predisposition and/or tumor progression [31,38–40]. Furthermore, it is proposed that an excess of DNA polymerase β activity during a non-proliferative phase could compete with or substitute for error-free DNA polymerases [40,41]. Therefore, under circumstances where DNA replication was impaired directly or indirectly, the activity of DNA polymerase β may become predominant. In fact, we have recently observed that 8-oxo-dG inhibits Ras-MEK/ERK pathway in KG-1 cells and thus blocks progression to S phase [29], leading to the non-proliferating status. Thus, under such non-proliferating status in 8-oxo-dG-treated KG-1 cells, error-free DNA synthesis is diminished and instead DNA polymerase β promotes the incorporation of mutagenic nucleotides including endogenous 8-oxo-dGTP into DNA. Unfortunately, however, KG-1 cannot cope with the increase of 8-oxo-Gua level in DNA due to mutational loss of OGG1 activity. The resulting accumulation of 8-oxo-Gua in DNA may interfere with DNA chain elongation and transcription, and trigger DNA damage signaling linking to apoptosis and cell death. This may be a common process that cells respond to DNA damage [42]. Furthermore, the activity of other repair enzymes for 8-oxo-Gua in DNA would be reduced during this cell death process, thus stimulating the amplification of 8-oxo-Gua level in DNA. Our proposed model is supported further by the recent reports showing that cells which overexpress DNA polymerase β are more sensitive to apoptosis in response to ionizing radiation [43] or oxidative damage [44].

In our systems, the activity of DNA polymerase β was enhanced in KG-1 cells treated with exogenous 8-oxo-dG although its expression was constant. The possible explanation may be an extent of post-translational modification of DNA polymerase β, presumably mediated by poly(ADP-ribose) polymerase (PARP). It has been reported that PARP is associated with DNA polymerase α and reduces its activity via poly(ADP-ribosyl)ation [45]. DNA polymerase β is also physically associated with a base excision repair complex which consists of PARP, XRCC1 and DNA ligase III [46]. Therefore, it is assumed that DNA polymerase β may also be poly(ADP-ribosyl)ated and its activity decreases. However, under the apoptosis of 8-oxo-dG-treated KG-1 cells, cleavage of PARP occurs [17], which may keep DNA polymerase β more active due to a low level of poly(ADP-ribosyl)ation. Instead, the expression of DNA polymerase α is suppressed via G1 arrest and thus its suppressed expression overrides the enhanced activity followed by PARP cleavage. Overall, a post-translational modification as well as non-proliferating status may potentiate the incorporation of 8-oxo-dGTP into DNA by DNA polymerase β.

The most well-known action of 8-oxo-Gua in DNA is promutagenic due to its property to mismatch with adenine instead of cytosine causing transversion of GC to TA [2,3]. In addition, it has been demonstrated that 8-oxo-Gua in DNA has oxidative-damage inducing effect [4,5] and triggers cell cycle arrest and apoptosis [17]. However, because free deoxyguanocine (dG) is most susceptible to oxidative attack compared with other deoxynuceo-

sides such as dA, dC and dT [5], cells upon oxidative insult may have a high level of 8-oxo-dG in cytosol. Therefore, studies have been done to explore cellular functions of 8-oxo-Gua-containing molecules in cytosol. First, 8-oxo-Gua-containing molecules in cells may not be simple oxidative by-products but an active molecules able to initiate cell signaling. It has been shown in vivo that KG-1 cells treated with 8-oxo-dG shows Ras inactivation and up-regulation of p21, a cell cycle-inhibiting protein [29]. In addition, it has also been shown in vitro that 8-oxo-GTP affects the activities of small GTP-binding proteins such as Ras, Rac1 and Cdc42 [47]. Second, in this study, 8-oxo-dG is suggested to have an anti-cancer activity to OGG1-deficeint cancer cells. Most of nucleoside analogues used as anticancer drugs exert cytotoxicity to any cells including normal cells, while 8-oxo-dG shows its selectivity to OGG1-deficient cells. However, the clinical usefulness of 8-oxo-dG as an anticancer agent depends on the occurrence rate of OGG1-deficient cancer cells.

It is still questionable how exogenous 8-oxo-dG activates Ras signaling pathway only in KG-1 and other cells with low OGG1 activity. We have to answer whether this activity of 8-oxo-dG are related to the deficiency of OGG1 function or whether cells like KG-1 have sensor molecule(s) that recognize 8-oxo-dG in cytosol and trigger cell signaling when they bind this molecule(s). The studies to answer these questions will shed light on the mechanism underlying the cellular effects of 8-oxo-dG.

Acknowledgments

We are grateful to all members of Dr. Chung's laboratory. This work was supported by the Ministry of Science and Technology of Korea through the National Research Laboratory (NRL) for Free Radicals.

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